Oral Science International 12 (2015) 5–8

Contents lists available at ScienceDirect

Oral Science International

journal homepage: www.elsevier.com/locate/osi

Original Article

Occurrence of *Helicobacter pylori* in saliva from preschool-age children

Jun Ueda*, Akira Yamaguchi, Kohichi Shibasaki

Department of Oral & Maxillofacial Surgery and Systemic Medicine, Graduate School of Life Dentistry at Niigata, The Nippon Dental University, Niigata, Japan

ARTICLE INFO

Article history:
Received 25 April 2013
Received in revised form 9 September 2014
Accepted 10 September 2014

Keywords:
H. pylori
Saliva
Primary infection
Preschool-age children
RT-PCR

ABSTRACT

Introduction: It is known that initial infection with *Helicobacter pylori* can occur in children aged 5 years or less, but there have been no studies investigating the period of initial infection. Here, 418 children attending preschool, and their parents, were tested for *H. pylori* DNA by real-time PCR using saliva samples in order to infer the period of initial infection of *H. pylori*.
Methods: Subjects took samples of their saliva by chewing on a sterile absorbent cotton roll for 2 minutes, and samples were stored at −80°C until they were examined. DNA was extracted from 100 μl of saliva and was tested for *H. pylori* DNA using the TaqMan method.
Result: The *H. pylori* DNA detection rate among children was: 4.1% among 3-year-olds, 4.9% among 4-year-olds, 10.0% among 5-year-olds, and 13.3% among 6-year-olds. The detection rate thus increased with age, showing a sharp increase from age 4 to 5 years. The rate of *H. pylori* detection among mothers of *H. pylori*-positive children was 56.2%, which was significantly greater than the rate among mothers of *H. pylori*-negative children (17.2%).
Conclusion: These results suggest that initial infection occurs even in children aged under 3 years, and the rise in detection rate between age 4 and 5 years suggests that there is a high risk of *H. pylori* infection during this period. Furthermore, the finding that the positivity rate was significantly higher among mothers of *H. pylori*-positive children than mothers of *H. pylori*-negative children indicates that mother-child infection is the most important route.

1. Introduction

Helicobacter pylori is a Gram-negative, microaerophilic bacterium with powerful urease activity and a helical structure. It was first isolated and identified in 1983 by Warren and Marshall [1], who found it in the gastric mucosa of patients with gastritis. *H. pylori* breaks down urea with urease, producing ammonia. As this neutralizes the gastric acid, the bacterium is capable of living in a strongly acidic environment. When present in the stomach, *H. pylori* often inhabits the mucus layer or gastric pits and may attach firmly to the surface of epithelial cells [2–4]. Numerous epidemiological studies have shown *H. pylori* infection to be a cause of acute and chronic gastritis, as well as gastric and duodenal ulcers, and also to be involved in the onset of stomach cancer and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma [5–16]. Recent studies have shown a considerable number of cases of idiopathic thrombocytopenic purpura (ITP) and childhood anemia associated with *H. pylori* infection [17–19], and improvement of symptoms as a result of *H. pylori* elimination has been reported [20,21]. Given the current emphasis on preventive medicine, grasping the status of *H. pylori* infection is essential.

The period of initial infection with *H. pylori* is reported to be most common up to 15 years of age [22,23]. With regard to the infection route, *H. pylori* DNA has been demonstrated in saliva and plaque, and infection associated with mouth disease, from which direct oral–oral infection can occur, is regarded as the most likely route [24–27]. In addition, infection from ingesting water or food contaminated with bacteria is believed to be an infection route, as a close relationship has been found between the *H. pylori* infection rate and the state of the sanitation environment, such as water and sewer services [25]. With regard to oral–oral infection, Sugiura et al. [28] investigated *H. pylori* infection status among 92 children attending a pediatric clinic and their parents, and analyzed bacterial DNA in order to subtype it. They reported that close contact between the mother and child during infancy is a major cause

* Corresponding author at: Department of Oral & Maxillofacial Surgery and Systemic Medicine, Graduate School of Life Dentistry at Niigata, The Nippon Dental University, 1-8 Hamauracho, Chu-o-ku, Niigata 951-8580, Japan.
Tel.: +81 025 267 1500; fax: +81 025 267 1582.
E-mail address: uenne@ngt.ndu.ac.jp (J. Ueda).

http://dx.doi.org/10.1016/S1348-8643(14)00028-7
1348-8643/

of initial infection. However, there have been few epidemiological studies of infection in young children [19,29,30], and all of these have used immunoassays to detect *H. pylori* antigens in feces or urine. Here, we detected and examined *H. pylori* DNA in the saliva of children and their parents, in order to infer the period of initial *H. pylori* infection.

2. Materials and methods

2.1. Subjects

The subjects were 418 children aged 3–6 years attending one of two kindergartens or one nursery school in Niigata City, Niigata Prefecture, Japan, and their parents (338 mothers and 284 fathers). All samples that were in suitable condition and quantity for examination were examined, and no data were excluded. Subjects were given a full explanation of the aims and the content of the study, and they gave their consent to participate. This study was carried out with the approval of the Ethics Committee of Nippon Dental University School of Life Dentistry at Niigata.

2.2. Methods

2.2.1. Collection and storage of samples

In principle, the collection of salivary samples was carried out at subjects' homes, after they woke up in the morning and before they cleaned their mouths. Salivary samples from children were collected by their parents, and parents collected their own samples. The samples were taken by placing a sterile absorbent cotton roll in the mouth and chewing once a second for 2 min. The cotton roll was then hermetically sealed in a plastic bag for collection. In a laboratory, the saliva was collected from the absorbent cotton roll by pressure using a 10-ml syringe, and the sample was stored at −80 °C until examination.

2.2.2. Extraction of H. pylori DNA from saliva

The saliva was thawed at room temperature, and 100 μl of phosphoric acid buffer solution (pH 7.4) was added to 100 μl of saliva. DNA was extracted using a QIAamp Mini Kit (Qiagen Ltd., Crawley, UK). DNA was refined by ethanol precipitation and the sample was dissolved in 15 μl of TE buffer solution (pH 8.0).

2.2.3. H. pylori DNA detection using real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was carried out by the TaqMan method using 5 μl of extracted *H. pylori* DNA solution as the template with 12.5 μl UMM (Fast Universal PCR Master Mix; Applied Biosystems Japan, Ltd., Tokyo, Japan). Primers were *pylori*-f/r [31] and the probe was *pylori*-TP (Table 1). The reaction solution was made up to a final volume of 25 μl with sterile distilled water, and real-time PCR carried out on a Step One Real-Time PCR System (Applied Biosystems Japan, Ltd., Tokyo, Japan). *H. pylori* DNA purified from a colony found by culture to be positive for *H. pylori* was used as a positive control, and sterile distilled water was used as a negative control.

Table 1
Sequences of primers and probes using real-time PCR.

	Sequence (5′ → 3′)	Dye, no. of bases
Primer		
pylori-F	5′-GGGTATTGAAGCGATGTTTCCT	FAM
pylori-R	5′-GCTTTTTTGCCTTCGTTGATAGT	22
Probe		
pylori-TP	5′-CTCGTAACCGTGCATAC-3′ MGB	23

Table 2
H. pylori DNA detection rate among children.

Age	3 years	4 years	5 years	6 years
No. of subjects	73	185	130	30
DNA detection rate	4.1%	4.9%	10.0%	13.3%

2.2.4. Protection of subjects' personal data

From a standpoint of protection of personal data, the study was performed in such a way as to ensure that the members of staff of participating facilities and researchers involved in the study were unable to identify personal data such as the name or test results of any of the subjects. All children and parents were numbered by family, and samples were labeled with the family number and child and parent number, and were taken to the laboratory by a staff member from the facility. Similarly, the test results were placed in numbered envelopes in a single batch and returned to the families by the participating facilities.

2.2.5. Statistical processing

The *H. pylori* detection rate, children's age group, parents' age group, and parent–child concordance were tested using the χ^2 test, with the level of significance set at $P < 0.05$.

3. Results

3.1. H. pylori DNA detection rate among children

There were 418 children, of whom 73 were aged 3 years, 185 were aged 4 years, 130 were aged 5 years, and 30 were aged 6 years. The *H. pylori* DNA detection rates were as follows: 3 years, 4.1%; 4 years, 4.9%, 5 years, 10.0%; and 6 years, 13.3%. While the detection rate increased with age, the difference was not significant (Table 2).

3.2. H. pylori DNA detection rate among mothers

There were 338 mothers, of whom 13 were in their 20s, 233 in their 30s, 90 in their 40s, and two in their 50s. The *H. pylori* DNA detection rates were as follows: 20s, 23.1%; 30s, 22.3%, 40s, 13.3%; and 50s, 50.0%. There were no significant differences between age groups (Table 3).

3.3. H. pylori DNA detection rate among fathers

There were 284 fathers, of whom four were in their 20s, 178 in their 30s, 98 in their 40s, and four in their 50s. The *H. pylori* DNA detection rates were as follows: 20s, 0.0%; 30s, 8.4%; 40s, 7.1%; and 50s, 0.0%. There were no significant differences between age groups (Table 3). However, the detection rate among fathers in their 30s was significantly lower than the 22.3% rate for mothers in their 30s ($P < 0.01$), and a similar trend was seen in the 40s age group.

3.4. Relationship between H. pylori DNA detection in children and their parents

Of the 25 children who tested positive for *H. pylori* DNA, in 13 cases (52%) only the mother tested positive, in three cases (12.0%) only the father, and in one case (4.0%) both parents. This means

Table 3
H. pylori DNA detection rate among parents.

Age	20s	30s	40s	50s
No. of mothers	13	233	90	2
DNA detection rate	23.1%	22.3%	13.3%	50.0%
No. of fathers	4	178	98	4
DNA detection rate	0.0%	8.4%	7.1%	0.0%

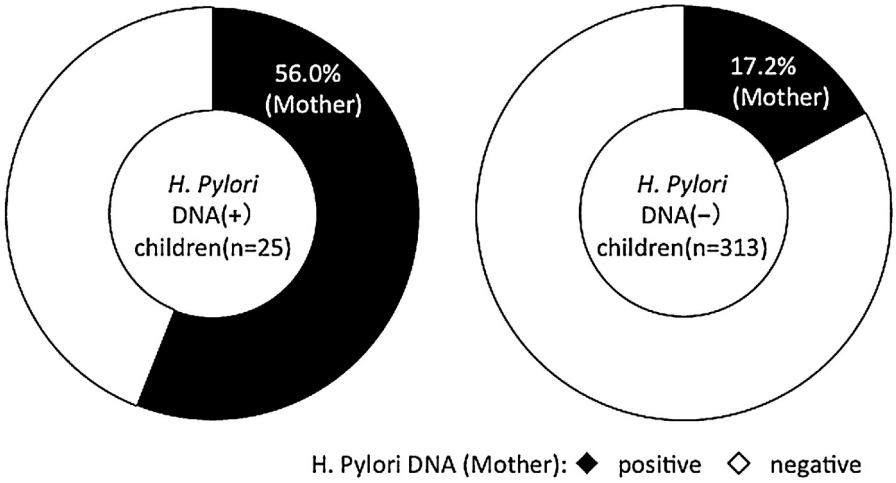

Fig. 1. Positive concordance rate for *H. pylori* DNA in mother and child. The filled area is the concordance of mothers who tested positive for *H. pylori* DNA. Among the 338 cases in which samples were taken from both mother and child, 25 of the children tested positive for *H. pylori* DNA and 14 of their mothers (56%) also tested positive. In comparison, 313 children tested negative for *H. pylori* DNA and a lower proportion of their mothers (54 mothers, 17.2%) also tested negative, showing a significant difference between groups ($P < 0.05$).

that, of the 25 children who tested positive, one or both parents tested positive for *H. pylori* DNA in 17 cases (68.0%). The *H. pylori*-positive concordance rate was higher between mother and child than between father and child, and the mother–child relationship was investigated. Among the 338 cases in which samples were taken from both mother and child, 25 of the children tested positive for *H. pylori* DNA and 14 of their mothers (56%) also tested positive. In comparison, 313 children tested negative for *H. pylori* DNA and a lower proportion of their mothers (54 mothers, 17.2%) also tested negative, showing a significant difference between groups ($P < 0.05$) (Fig. 1).

4. Discussion

H. pylori infection is reported to have a high incidence rate in developing countries and a low rate in developed countries [32]. In Japan, there is a biphasic pattern: the infection rate among people aged ≤20 years is approximately 20%, or roughly the same as in major developed countries, but among people aged ≥50 years, it is high at 60% or higher [22]. The infection rate is high in developing countries that do not have adequate water and sewer services or have a poor sanitation environment [19,32], and reports from China [33] and Russia [34], where there has been remarkable economic growth and the sanitation environment is being improved, show a biphasic pattern of infection similar to Japan. These findings provide evidence for a close relationship between social environment, including sanitation, and *H. pylori* infection rate.

The *H. pylori* infection route is believed to be oral, and is probably oral–oral infection between mothers and children and infection from contaminated water. However, there have been few detailed studies of the period of initial *H. pylori* infection and the infection route. We previously reported that *H. pylori* from the stomach and from the mouth have the same genotype at the molecular biological level, and that approximately 50% of mothers of *H. pylori*-positive children have a matching *H. pylori* DNA subtype [28,35], showing that *H. pylori* infection can be diagnosed by testing *H. pylori* DNA in the saliva.

In the present study, we tested for *H. pylori* DNA using real-time PCR on samples of saliva from children aged 3–6 years attending preschool in order to investigate the period of initial infection during infancy. With regard to *H. pylori* infection in children, Naito et al. [36] measured *H. pylori* immunoglobulin G (IgG) in the urine of 452 children aged 4–10 years and reported a detection rate of 4.0–6.7% with no differences due to age. In the present study, the *H. pylori* DNA detection rate rose somewhat with age, but there were no significant differences. However, the rate was 4.9% among 4-year-olds and 10.0% among 5-year-olds, which is a sharp increase. At 3 years, 4.1% of children were already infected with *H. pylori*, suggesting that the first infection is before the age of 3 years, and the increase at 4–5 years suggests that there is a greater risk of infection during this period. At 4–5 years, children are developing socially and their sphere of activity is expanding, so it is likely that they have increased chances of coming into contact with people other than their parents and contaminants that present the risk of infection. The possibility of infection by this route cannot be ignored. There is a need for future studies with sampling methods that are feasible for examining children under the age of 3 years and also for follow-up studies by age.

The *H. pylori* DNA detection rate among adults has been reported to increase with age [37], but in the present study the detection rate among the parents showed no significant differences by age group. A possible reason for this is that there were very few samples from parents in their 20s (13 mothers and four fathers) and in their 50s (two mothers and four fathers). Kumagai et al. [38] and Asaka et al. [37] report that the *H. pylori* infection rate among Japanese adults during the 1990s was ≥40% for people in their 30s and ≥60% for people in their 40s. In the present study, the infection rates were 22.3% for mothers and 8.4% for fathers in their 30s, and 13.3% for mothers and 7.1% for fathers in their 40s. The infection rates were thus lower in both age groups, which appear to indicate that the *H. pylori* infection rate among Japanese adults has fallen in recent years.

Although there are generally believed to be no gender differences in the *H. pylori* infection rate, in the present study, the *H. pylori* DNA detection rate among mothers in their 30s was 22.3%, compared to which the rate among fathers in their 30s (8.4%) was significantly lower ($P < 0.01$). A similar trend was seen among parents in their 40s. The reason for this is not clear, but as saliva was collected for 2 min after getting up in the morning before rinsing the mouth or cleaning the teeth in principle, it is possible that working fathers, who tend to rush in the mornings, were less able to collect sufficient saliva.

With regard to the relationship between *H. pylori* DNA detection in children and in their parents, Sugiura et al. [28] found that in all cases where *H. pylori* DNA was detected in the saliva of a

child, it was also detected in the saliva of either one or both parents. In the present study, however, 25 children tested positive for *H. pylori* DNA, and in eight of these cases (32.0%) neither parent tested positive. It is possible that there are infection routes other than parents (e.g., other family members, classmates, etc.), or that saliva was not correctly sampled, and this is an issue for future study. However, the *H. pylori* DNA detection rate in mothers of *H. pylori*-positive children was significantly higher than in mothers of *H. pylori*-negative children, showing the predominance of *H. pylori* infection transmitted from mothers to children.

In the present study, a follow-up of specific samples was not possible because anonymity was preserved by assigning numbers to facilities and subjects for reasons of protection of personal data. In future studies, the method of sample collection and the method of ensuring anonymity will need to be reconsidered. It is hoped that *H. pylori* infection prevention awareness programs aimed at parents and at children will be bolstered, and also that the effectiveness of infection prevention interventions in the sanitation environment of kindergartens and nursery schools will be investigated.

5. Conclusions

Children attending preschool and their parents were tested for *H. pylori* DNA using real-time PCR on samples of saliva, and the following results were obtained:

1. *H. pylori* DNA was detected in children aged 3 years, suggesting that initial infection may be at <3 years of age.
2. The *H. pylori* DNA detection rate increased with increasing age from 3 to 6 years, with a sharp increase between 4 and 5 years, thus suggesting that there is a high risk of infection at 4–5 years.
3. The *H. pylori* DNA detection rate was significantly higher among mothers of *H. pylori*-positive children than among mothers of *H. pylori*-negative children, indicating that infection from the mother is the most important route.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors wish to express their gratitude to Niigata Seiryo Kindergarten, Niigata Ariake Kindergarten, and Niigata Sasaguchi Nursery School, as well as to all the families who kindly cooperated with this study by providing samples.

References

[1] Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983;4:1273–5.
[2] Dunn BE, Campbell GP, Perez-Perez IP, et al. Purification and characterization of urease from *Helicobacter pylori*. *J Biol Chem* 1989;265:9464–9.
[3] Marshall BJ, Goodwin CS, Warren JR, et al. Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori*. *Lancet* 1998;2:1437–42.
[4] Gulisano M, Marceddu S, Tanda F, et al. Morphologic study of the gastric mucosa during *Helicobacter pylori* infections. *Ital J Anat Embryol* 1992;97:229–38.
[5] Hentschel E, Brandstatter G, Dragosics B, et al. Effect of ranitidine and amoxicillin plus metronidazole on the eradication of *Helicobacter pylori* and the recurrence of duodenal ulcer. *N Engl J Med* 1993;328:308–12.
[6] NIH Consensus Conference. *Helicobacter pylori* in peptic ulcer disease. NIH consensus development panel on *Helicobacter pylori* in peptic ulcer disease. *JAMA* 1994;272:65–9.
[7] BMJ Publishing Group Ltd. and British Society of Gastroenterology. Current European concepts in the management of *Helicobacter pylori* infection. The Maastricht Consensus Report. European *Helicobacter pylori* Study Group. *Gut* 1997;41:8–13.
[8] Rene WM, Erik EA, Brican K, et al. Prevention of ulcer recurrence after eradication of *Helicobacter pylori*: a prospective long-term follow up study. *Gastroenterology* 1997;113:1082–6.
[9] Lam SK, Talley NJ. *Helicobacter pylori* Consensus, report of the 1997 Asian Pacific Consensus Conference on the management of *Helicobacter pylori* infection. *J Gastroenterol Hepatol* 1998;13:1–12.
[10] Asaka M, Satoh K, Sugano K, et al. Guidelines in the management of *Helicobacter pylori* infection in Japan. *Helicobacter* 2001;6:177–86.
[11] Asaka M, Sugiyama T, Kato M, et al. A multi-center, double-blind study on triple therapy with lansoprazole, amoxicillin and clarithromycine for eradication of *Helicobacter pylori* in Japanese peptic ulcer patients. *Helicobacter* 2001;6:254–61.
[12] Malfertheiner P, Megraud F, O'Morain C, et al. Current concepts in the management of *Helicobacter pylori* infection – the Maastricht 2 – 2000 Consensus Report. *Aliment Pharmacol Ther* 2002;16:167–80.
[13] Marshall BJ, Armstrong JA, David B, et al. Attempt to fulfil Koch's postulates for pyloric Campylobacter. *Med J Aust* 1985;142:436–9.
[14] Watanabe T, Tada M, Nagai H, et al. *Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils. *Gastroenterology* 1998;115:642–8.
[15] Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, et al. *Helicobacter pylori* associated gastritis and primary B-cell gastric lymphoma. *Lancet* 1991;338:1175–6.
[16] Terai S, Iijima K. Long term outcomes of gastric mucosa associated lymphoid tissue lymphomas after *Helicobacter pylori* eradication therapy. *Tohoku J Exp Med* 2008;214:79–87.
[17] Suogle OD, Gokce S, Saglam AT. Association of *Helicobacter pylori* infection with gastroduodenal disease, epidemiologic factors and iron-deficiency anemia in Turkish children undergoing endoscopy, and impact on growth. *Pediatr Int* 2007;I49:858–63.
[18] Akcam S, Ozdem S, Yilmaz A. Serum ferritin, vitamin B12, folate, and zinc levels in children infected with *Helicobacter pylori*. *Dig Dis Sci* 2007;52:405–10.
[19] DiGirolamo AM, Perry GS, Gold BD. *Helicobacter pylori*, anemia, and iron deficiency relationships explored among Alaska native children. *Pediatr Infect Dis J* 2007;26:927–34.
[20] Jackson SC, Beck P, Buret AG. Long term platelet responses to *Helicobacter pylori* eradication in Canadian patients with immune thrombocytopenic purpura. *Int J Hematol* 2008;88:212–8.
[21] Ando K, Shimamoto T, Tauchi T, et al. Can eradication therapy for *Helicobacter pylori* really improve the thrombocytopenia in idiopathic thrombocytopenic purpura? Our experience and a literature review. *J Hematol* 2003;77:239–44.
[22] Haruma K. Trend toward a reduced prevalence of *Helicobacter pylori* infection, chronic gastritis, and gastric cancer in Japan. *Gastroenterol Clin North Am* 2000;29:623–31.
[23] Banatvala N, Mayo K. The cohort effect and *Helicobacter pylori*. *J Infect Dis* 1993;168:219–21.
[24] Marshall B, Howat AJ, Wright PA, et al. Oral fluid antibody detection in the diagnosis of *Helicobacter pylori* infection. *J Med Microbiol* 1999;11:1043–6.
[25] Adler I, Denninghoff VC, Alvarez MI, et al. *Helicobacter pylori* associated with glossis halitosis. *Helicobacter* 2005;4:312–7.
[26] Olsson K, Wadstrom T, Tyszkiewicz T, et al. *H. pylori* in dental plaques. *Lancet* 1993;341:956–7.
[27] Rasmussen LT, Labio RW, Gatti LL, et al. *Helicobacter pylori* detection in gastric biopsies, saliva and dental plaque of Brazilian dyspeptic patients. *Mam Inst Oswaldo Cruz* 2010;105:326–30.
[28] Sugiura H, Yamaguchi A, Shibasaki K, et al. Study of intrafamilial transmission of *Helicobacter pylori* infection in Japanese children. *J Jpn Stomatol* 2006;55:108–12.
[29] Okuda M, Miyashiro E, Koike M, et al. Breast-feeding prevents *Helicobacter pylori* infection in early childhood. *Pediatr Int* 2001;43:714–5.
[30] Kato S, Tachikawa T, Ozawa K, et al. Urine-based enzyme-linked immunosorbent assay for the detection of *Helicobacter pylori* infection in children. *Pediatrics* 2001;107:87.
[31] McDaniels E, Wymer L, Rankin C, et al. Evaluation of quantitative real time PCR for the measurement of *Helicobacter pylori* at low concentrations in drinking water. *Water Res* 2005;39:4808–16.
[32] Aoki K, Kihaile PE, Castro M, et al. Seroprevalences of *Helicobacter pylori* infection and chronic atrophic gastritis in the United Republic of Tanzania and the Dominican. *Health Prev Med* 2004;9:170–5.
[33] Chen J, Xiao LB, Wang QT, et al. Decreasing seroprevalence of *Helicobacter pylori* infection during 1993–2003 in Guangzhou, Southern China. *Helicobacter* 2007;12:164–9.
[34] Tkachenko MT, Zhannat NZ, Erman LV, et al. Dramatic changes in the prevalence of *Helicobacter pylori* infection during childhood: a 10-year follow-up study in Russia. *J Pediatr Gastroenterol Nutr* 2007;45:428–32.
[35] Yamada R, Yamaguchi A, Shibasaki K. Detection and analysis of *Helicobacter pylori* DNA in the gastric juice, saliva, and urine by nested PCR. *Oral Sci Int* 2008;5:24–34.
[36] Naito Y, Shimizu T, Haruna H, et al. Changes in the presence of urine *Helicobacter pylori* antibody in Japanese children in three different age groups. *Pediatr Int* 2008;50:291–4.
[37] Asaka M, Kimura T, Kudo M, et al. Relationship of *Helicobacter pylori* to serum pepsinogens in an asymptomatic Japanese population. *Gastroenterology* 1992;102:760–6.
[38] Kumagai T, Malaty H, Graham D, et al. Acquisition versus loss of *Helicobacter pylori* infection in Japan: results from an 8-year birth cohort study. *J Infect Dis* 1998;178:717–21.